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Minireview

Seed recalcitrance — current perspectives

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The phenomenon of non-orthodox seed behaviour is presented in the framework of the probability of being much more common than might be expected, considering that scientific knowledge about seeds is largely drawn from what has been established for a mere handful of cultivated crop species. The scattering of species producing recalcitrant seeds across most angiosperm families (and among the gymnosperms) appears to have no taxonomic basis, although recalcitrance might be the ancestral seed condition. Recalcitrance is considered not as deviant, but rather as one extreme of a continuum of seed behaviour based on the response to dehydration, the other extreme being manifested by those orthodox seeds that are able to tolerate almost total desiccation. Post-harvest seed responses to dehydration are presented in the context of developmental events that characterise pre-shedding development. Those that acquire desiccation tolerance and will survive lengthy periods in the desiccated state do so as a result of the full development and interaction of a suite of mechanisms enabling this competence. Highly recalcitrant seeds which consistently remain metabolically active, are considered not to possess, or express, most or all of these, while inbetween the extremes of recalcitrance and orthodoxy, seeds that might be considered as non-orthodox (but also not recalcitrant) will manifest these mechanisms and their interactions variably — thus accounting for a continuum of seed behaviour in the context of relative desiccation tolerance. Emphasis is laid on the fact that the degree of dehydration tolerated by seed tissues is a function of the rate of dehydration: the more rapidly water can be lost, the less time is available for metabolism-linked damage, and the lower the water content that can be attained without lethal injury. However, recalcitrant seeds cannot lose structure-associated (non-freezable) water without sustaining lethal injury, which is considered as desiccation damage *sensu stricto*. Finally, the limited potential for storage of recalcitrant seeds is discussed, and the alternative biotechnological approach of germplasm conservation by means of cryostorage of embryonic axes, is presented.

Introduction

There is a common perception that mature seeds of angiosperms, and indeed of the commonly-known gymnosperms, are shed as dry entities that are incapable of further development (usually germination) until an adequate water supply becomes available. In the dehydrated condition, such seeds can be stored for variable periods, which are predictable under defined seed water content and temperature conditions (Ellis and Roberts 1980). This type of storage behaviour is termed 'orthodox', a description applied to such seeds for some 30 years (Roberts 1973). Most of the accepted dogma about seed structure, development, physiology and germination has been derived from observations and in-depth investigations on orthodox seeds. However, almost all the amassed information has been derived from work on seeds of considerably fewer than 0.1% of all higher plants! This perhaps, is not surprising, considering that the seeds of around 20 important crops and those of a few woody species — all of which are orthodox — have been the major focus for most investigators.

With intensification of world-wide interest in regional indigenous plant species and biodiversity, however, there is steadily increasing evidence that orthodox seed behaviour might not be the norm. The ever-growing list of species producing non-orthodox seeds is an extension of the compendium of recalcitrant crop seed producers, already published by Chin and Roberts in 1980. At the same time that the term, orthodox, was introduced into seed biology, the descriptor, recalcitrant, was applied to those seeds shed at high water contents and having in common the phenomenon of desiccation-sensitivity (i.e. not being able to withstand removal of more than a small proportion of their tissue water, without rapidly lethal results). More accurately, it was because the seeds described could not be stored at low water contents under low relative humidity (RH) conditions, that they were termed recalcitrant (Roberts 1973).

Many seeds identified as being recalcitrant are produced

by tree species, especially those of tropical provenance. For example: *Artocarpus* spp. [Moraceae] (jackfruit; breadfruit); *Avicennia marina* [Avicenniaceae] (and other mangroves, e.g. *Bruguiera gymnorhiza* [Rhizophoraceae]); *Barringtonia racemosa* [Lecythidaceae] and other riparian species, as well as *Bertholletia excelsa* (Brazil nut); *Ekebergia capensis* and *Trichilia* spp. [Meliaceae]; *Garcinia* spp. [Clusiaceae (=Guttiferae)] (mangosteens); *Hevea brasiliensis* [Euphorbiaceae] (commercial/natural rubber); *Litchi chinensis* [Sapindaceae] (lychee); *Mangifera indica* [Anacardiaceae] (mango); *Persea americana* [Lauraceae] (avocado); *Shorea* spp. [Dipterocarpaceae] (e.g. sal [India]; engkabang [Malaysia]); *Theobroma cacao* [Sterculiaceae] (cocoa). Although many of these species are indigenous to mesic tropical regions where conditions continually favour seedling establishment, others occur in temperate regions with marked seasonality. Among temperate species producing recalcitrant seeds are *Aesculus hippocastanum* [Hippocastanaceae] (horse chestnut); *Acer* spp. [Aceraceae] (sycamore; sugar maple; silver maple); and *Quercus* spp. [Fagaceae] (e.g. pedunculate/English oak; red oak; i.a.); *Camellia sinensis* [Theaceae] (tea); *Poncirus trifoliata* [Rutaceae] (trifoliate orange). Production of recalcitrant seeds is not exclusive to dicotyledonous tree species: for example, such seeds are characteristic of *Landolphia kirkii* [Apocyanaceae] which is a woody (dicot) vine; several gymnospermous trees, e.g. *Podocarpus henkelii* (Henkel's yellowwood) and *Araucaria* spp. (monkey puzzle), as well as certain monocotyledonous species including *Cocos nucifera* [Palmae] (coconut); *Scadoxus* [*Haemanthus*] *membranaceus* [Amaryllidaceae] (dwarf paintbrush); *Zizania* spp. [Poaceae] (wild rice).

It can be seen from these examples (and there are many more), that the phenomenon of seed recalcitrance is widespread across families. Although there are families in which apparently no species produce recalcitrant seeds, there seems to be little pattern among the angiosperms regarding the occurrence of seed recalcitrance. Nevertheless, recalcitrance has been suggested to be the ancestral seed condition (Pammenter and Berjak 2000), and hence cannot be considered to be a manifestation of deviant behaviour. Rather, recalcitrant seeds might be thought to behave in an unexpected manner — but only because of entrenched perceptions that seeds are all similar to beans or wheat!

There are, however, marked differences between highly desiccation-tolerant orthodox seeds, at the one extreme, and those recalcitrant species which cannot tolerate any significant loss of water without lethal consequences (Berjak and Pammenter 1999). Even among orthodox species, not all can be as severely dehydrated as others (Walters 1998), and among recalcitrant types, there are notable differences in the proportion of water loss that can be tolerated under similar drying conditions (Farrant *et al.* 1989). Although a category of seeds that can withstand dehydration to relatively low water contents has been described as showing intermediate characteristics (Ellis *et al.* 1990, Hong and Ellis 1996), it is likely that when seed biology of a significantly greater number of angiosperm (and gymnosperm) species has been studied, a continuum of seed behaviour will emerge. This will be based not just on relative sensitivity or tolerance to desiccation, but also on a spectrum of other characteristics (Berjak and Pammenter 1994).

Although orthodox seed storage is not problem-free, adherence to guidelines for low RH (to maintain low seed water content) and temperature of the store will generally conserve vigour and viability for extended periods, provided the seeds are of good quality and sufficiently desiccated at the outset. Especially valuable seed (e.g. in base collections) is conventionally stored at -18°C. However, similar approaches are precluded for recalcitrant seeds which must remain hydrated to retain viability. The necessarily high seed water contents also preclude storage at below-zero temperatures and additionally, recalcitrant seeds of some tropical species are chilling-sensitive, being damaged at cold temperatures that are well above 0°C. For example, seeds of *Theobroma cacao* (cocoa) appear to be damaged at temperatures below the high value of 15°C (Chin and Roberts 1980). Recalcitrance thus poses major problems in terms of short- to medium-term storage for planting programmes and the seed trade, as well as necessitating a completely different approach for long-term germplasm conservation (see below).

Recalcitrance and seed development are inextricably linked

While the pre-shedding development of orthodox seeds is characterised by a third and final phase, maturation drying, during which dry mass accumulation ceases and seed water content and thus fresh weight, decline significantly, this phase is essentially absent in recalcitrant types. Although some small measure of decline in the absolute amount of tissue water may occur, what seems to dominate the developmental phase preceding shedding is that there is no cessation in the accumulation of dry matter (Hong and Ellis 1990, Farrant *et al.* 1992, Tompsett and Pritchard 1993, Finch-Savage and Blake 1994, Fu *et al.* 1994, Lin and Chen 1995), while the amount of water stays essentially constant. Consequently, water content expressed either as a percentage of the fresh mass (fmb) or on a dry mass basis (g water g^{-1} dry mass [dmb]) may appear to decline to a limited extent. However, the water content of recalcitrant seeds at shedding, is always high — from 0.3 to >4.0g g^{-1}, depending on the species, the season and the provenance, in comparison with that of mature orthodox seeds (generally 0.15g g^{-1} or less). On attempting to categorise unfamiliar seeds, water contents at shedding exceeding 0.3g g^{-1} give the first clue to their being recalcitrant.

However, seeds of some species that are not recalcitrant may be shed at similarly high water contents, but the factor that differentiates them is their response to desiccation. Methodical trials involving stepwise dehydration under strictly controlled conditions will result in vigour and viability loss after relatively little dehydration in recalcitrant seeds, while orthodox types will withstand desiccation to water contents as low as 0.053g g^{-1} (5% fmb). Seeds that fall into the category described as showing intermediate behaviour, will generally lose viability below water contents of 0.14–0.11g g^{-1} (12–10% fmb) (e.g. Hong and Ellis 1996).

Marked differences in desiccation-sensitivity exist among

recalcitrant seeds of different species, based on inherent properties such as the nature of the covering layers and seed composition. However, even within seeds of individual species, there is often notable inter- and intra-seasonal variation (Berjak *et al.* 1989, 1993). Such differences are dependent on both intrinsic properties of the seeds and the nature of conditions to which they are subjected — both prior to and after harvest. For experimental purposes, recalcitrant seeds are generally hand-harvested from the parent plant. As there are generally few indications of the relative imminence of natural shedding, the water content and developmental status of the seeds at harvest are among the intrinsic factors underlying variability. Additionally, when the seeds are subjected to dehydrating conditions, particularly the drying rate — and probably also the temperature — are critical factors that determine relative desiccation-sensitivity. As these parameters impose differences in response among seeds of individual species, it is apparent that there cannot be an unqualified 'critical [threshold] water content' below which seed viability will be lost (Pammenter *et al.* 1998). While the basis for this statement will be explored in greater depth below, it is important to note that a misleading feature of much of the published literature on individual recalcitrant seed species, is the identification of putative 'critical water contents'. However, in juxtaposition to this assertion, it must be realised that there will be a limiting water content below which retention of viability of seeds of individual species is impossible when dehydration is carried out, under even the most favourable conditions. This would be the critical water content sensu stricto, which is likely to be close to the level where only structure-associated (non-freezable) water persists (Pammenter *et al.* 1998, Walters *et al.* 2001). However, dehydration to such levels with concomitant retention of viability is highly unlikely to be achievable with intact seeds even under the most rapid drying conditions, simply because the rate of water loss would be far too slow (Pammenter *et al.* 1998).

The over-arching criterion determining recalcitrant behaviour is that the seeds are actively metabolic when they are shed or harvested (Berjak *et al.* 1989, Poulsen and Eriksen 1992, Lin and Chen 1995, Chien and Lin 1997, Farrant *et al.* 1997, reviewed by Côme and Corbineau 1996 and by Finch-Savage 1996), and remain so for a physiologically-defined period which varies from one species to another, provided there is no — or only minimal — water loss. When recalcitrant seeds are stored under conditions maintaining their water content (hydrated storage), depending on the species germinative metabolism will immediately be initiated, or developmental events grading into the onset of germination will occur (Berjak *et al.* 1989). The longevity of the seeds in hydrated storage is inversely related to the rate at which germinative metabolism proceeds: generally once cell division and extensive vacuolation occur during radicle extension, the seeds lose vigour and ultimately viability, because the requirement for additional water to support active growth cannot be met (Farrant *et al.* 1986, Berjak *et al.* 1989). Whether germinative metabolism ensues and proceeds rapidly or slowly, the inevitable consequence appears to be the imposition of a mild, but prolonged water stress which ultimately is lethal (Pammenter *et al.* 1994).

Two approaches to curtailing or delaying germinative metabolism in hydrated storage have suggested themselves: one is the use of relatively low storage temperatures — which is tenable in the case of seeds of temperate species (e.g. *Quercus robur* [Suzka and Tylkowski 1980]; *Araucaria hunsteinii* [Pritchard *et al.* 1995]), but does not provide a means for effective long-term storage, mostly because of the complicating factor of seed-associated fungi (see later). Cold-storage is also precluded for those (usually tropical) species that are chilling-sensitive (Chin and Roberts 1980). The other approach, *viz.* lowering seed water content to a level permitting ongoing vital metabolism but curtailing germination (storage at 'sub-optimal' water content) has been shown to be so injurious as to shorten seed lifespan relative to storage at the shedding water content (Drew *et al.* 2000). Those authors also showed that, at 'sub-optimal' water content, the seeds were over-run by proliferating fungi far more rapidly than were seeds stored without prior dehydration. Limited experimentation has shown the metabolic inhibitor, ABA, to be ineffective in curtailing germinative metabolism in storage, at least for cacao seeds (Mumford and Brett 1982).

When one considers that in most cases recalcitrant seeds are, in effect, seedlings (Berjak *et al.* 1989, 1996) then it is not surprising that they are no more amenable to these various manipulations, than would be seedlings or young plants grown from orthodox seeds. In order to understand — and perhaps be able to manipulate — recalcitrant seeds, a comprehensive appreciation of the factors that facilitate the unusual quality of desiccation-tolerance in orthodox seeds, is necessary.

What determines recalcitrant (or non-orthodox) vs orthodox seed behaviour?

There are no apparent differences in the progress of the phases encompassing histodifferentiation and reserve deposition during orthodox and recalcitrant seed development, and it is only after this that the patterns diverge (Farrant *et al.* 1993). However, what is not obvious from this overview, are the profound changes that occur accompanying the acquisition of desiccation-tolerance — i.e. the ability to withstand extreme dehydration — and the mechanisms that must become operative for orthodox seed survival in the desiccated state. It is the lack and/or only partial expression of the suite of interacting processes and mechanisms operative in orthodox seeds that precludes recalcitrant types from entering the third developmental phase — that of maturation drying — and therefore determines the basis of their unifying feature of desiccation-sensitivity (Pammenter and Berjak 1999). Recalcitrant behaviour is thus inevitably a product of seed development, explaining at least partly why manipulations to extend their post-harvest lifespan by imposing conditions to make them more like orthodox seeds, have been such a dismal failure.

Intracellular Physical Characteristics

A major characteristic of plant cells that are desiccation-tolerant, is their ability to withstand the strains that must follow

imposition of dehydration stress. A spectrum of cellular and intracellular features can be identified in orthodox seeds, which together enable the tissues to withstand the physical strains associated with dehydration. These include a significant reduction in the occurrence of large, fluid-filled vacuoles, which either break up into many, very much smaller bodies, and/or become filled with insoluble reserve material, usually in the form of protein. Additionally, in orthodox seeds, embryo cells characteristically accumulate large amounts of insoluble starch and/or lipid external to the vacuoles (reviewed by Vertucci and Farrant 1995). A further feature guarding against physical damage resulting from cell shrinkage during dehydration, is a plasticity of the walls that facilitates their folding (Webb and Arnott 1982). This spectrum of features, together with the ability of the cytoskeleton to dissociate in an orderly manner, militate against physical damage when water loss from the cells brings about their marked volume reduction in orthodox seeds. Additionally, although direct data are lacking, it is implicit that a conformation of the chromatin is assumed that will protect the integrity of the genome in the desiccated condition in orthodox seeds (Brunori 1967, Osborne and Boubriak 1994). Accompanying this, the nucleoskeleton which determines nuclear architecture (Moreno Díaz de la Espina 1995) and imposes intra-nuclear functional domains in the hydrated condition (Wolfe 1993), must be modified in a strictly controlled manner that will ensure its fully functional reconstitution when water once again becomes available.

Many of these characteristics, which are developmental features preceding or accompanying maturation drying in orthodox seed tissues, are know to be lacking, or to be manifested only partially, in the embryo cells of recalcitrant seeds. Even from the relatively few studies that have been done, there appears to be a correlation between the degree of recalcitrance, and the manifestation of some of these features. For example, in the highly recalcitrant (extremely desiccation-sensitive) seeds of the tropical species, *Avicennia marina*, embryo cells remain massively vacuolated and accumulate little by way of insoluble reserves (Farrant *et al.* 1997). Embryo cells of another tropical species, *Ekebergia capensis*, although accumulating a substantial amount of starch within plastids, also remain highly vacuolated (Pammenter *et al.* 1998): these seeds, while tolerating a somewhat greater degree of dehydration than those of *A. marina*, are nevertheless considerably more desiccation-sensitive than those of other recalcitrant species in which embryo cells become less vacuolated. As an example, seeds of the temperate species, *Aesculus hippocastanum*, which are relatively less recalcitrant, accumulate protein in small vacuoles and lay down starch in plastids and some lipid in extra-vacuolar sites in embryo cells (Farrant *et al.* 1997). Nevertheless, *A. hippocastanum* seeds are desiccation-sensitive, indicating that these microscopically-visible events alone are not effective devices to counteract the strains associated with dehydration. These intracellular features — which are highly conspicuous developments providing physical buffering against volume changes on dehydration in orthodox embryos — can be seen to be lacking, or only to be partially effective, in recalcitrant embryos, seemingly in line with their degree of desiccation-sensitivity.

Intracellular De-differentiation and Metabolic 'Switch Off'

A few studies in the 1960s and 1970s already described intracellular de-differentiation to accompany the onset of maturation drying in orthodox seeds, with the reversal of this process, *viz.* re-differentiation, occurring upon rehydration early during germination (Bain and Mercer 1968, Klein and Pollock 1968, Hallam 1972). In their 1997 study, Farrant *et al.* showed that organelle de-differentiation actually precedes this phase, occurring as a developmental event prior to, rather than triggered by, water loss. De-differentiation of mitochondria and plastids implies minimisation of their metabolic activity, which, if proceeding during dehydration, could yield a source of potentially uncontrolled free radicals resulting in serious to lethal intracellular damage (see later). In tandem with organelle de-differentiation, respiratory decline (i.e. metabolic 'switch off') has been shown to occur ahead of the maturation drying phase in *Phaseolus vulgaris* (Farrant *et al.* 1997), extending earlier studies associating a sharp decline in respiratory substrates and a fall in respiration rate with maturation drying (Rogerson and Matthews 1977).

Intracellular de-differentiation is not restricted to mitochondria and plastids: for example, endomembranes such as the rough ER become substantially reduced and the cisternae of Golgi bodies are dis-associated (Bain and Mercer 1966, Klein and Pollock 1968, Hallam 1972, reviewed by Vertucci and Farrant 1995). This implies not only the shut-down of membrane synthesis and processing, but also the reduction of membrane surface area and hence of sites that would undergo substantial, often deleterious, changes upon desiccation. A further indication of metabolic 'switch off' already described by Brunori in 1967, is the cessation of DNA replication and the arrest of most embryo cells in the G1 (pre-replication) phase in orthodox embryos with the onset of maturation drying. During G1, the DNA is in the undoubled form, described as 2C.

What is the status of such cryptic developmental events in recalcitrant seeds? In none of the recalcitrant embryos (of a variety of different species) examined electron microscopically, does marked de-differentiation occur. A comparison of the highly-recalcitrant embryos of *Avicennia marina* with those of *Aesculus hippocastanum*, which is less recalcitrant, showed that mitochondria occupied a greater proportion of the cytoplasmic volume in the more desiccation-sensitive species (Farrant *et al.* 1997). However, in both *A. marina* and *A. hippocastanum* the mitochondria remained highly differentiated, showing abundant cristae and dense matrices in keeping with the relatively high respiratory rates measured. Respiration rates are generally relatively high across a divergent species-spectrum of recalcitrant seeds (Poulsen and Eriksen 1992, Lin and Chen 1995, Chien and Lin 1997, and reviewed in 1996 by Côme and Corbineau and by Finch-Savage). There are insufficient data for cell cycling in recalcitrant embryos to comment unequivocally. However, in highly-recalcitrant *A. marina* seeds, which are poised for immediate germination after abscission from the parent tree, only the most transient cessation of DNA replication occurs, with resumption of DNA synthesis (the S phase) resulting in

its entering the more vulnerable, doubled state (4C), soon after shedding (Boubriak *et al.* 2000). This event occurs without an extraneous water source — i.e. at the seed water content typifying the newly-shed condition, which is quite unlike the G1 arrest reported for orthodox seeds entering maturation drying. In the temperate recalcitrant species, *Acer pseudoplatanus*, on the other hand, >60% of the embryo cells have been reported to be arrested in the 2C state, which might, however, be associated with the dormancy of these seeds in the newly-shed state (Finch-Savage *et al.* 1998). For mature seeds of *Azadirachta indica* characterised as intermediate, Sacandé *et al.* (1997) have shown that DNA occurs almost exclusively in the 2C state. Thus information on cell cycling in the few species of non-orthodox seeds studied has certainly not produced unequivocal results, and the survey needs to be extended considerably. However, the work on cell cycling in *A. marina* embryos from newly-shed seeds (in which the S-phase leading to the 4C condition is rapidly entrained) has shown that the DNA is severely damaged by only a slight degree of dehydration, which is a further indication of the vulnerability of actively metabolic subcellular components to water loss (Boubriak *et al.* 2000).

Antioxidant Systems

Free radicals, which are highly reactive, electron-seeking chemical species, are naturally produced during normal metabolism (e.g. the oxidised forms of the cytochromes are free radicals). However, production of these and other free radicals is under strict control and vegetative tissues contain a range of both enzymatic and non-enzymatic antioxidants which function to prevent injurious consequences of 'escaped' free radicals, the best known of which is peroxidation of membrane lipids (reviewed by Hendry 1993). This is likely to lead to membrane lipid lateral phase separation (literally a change from the more fluid, liquid crystalline state to the more rigid gel state) accompanied by protein displacement from the membranes (reviewed by Vertucci and Farrant 1995).

It is particularly when metabolism has the potential to become disturbed — as is the case when dehydration of plant tissues occurs — that free radical scavengers must be optimally active. Although metabolic processes appear to be curtailed at the onset of maturation drying in orthodox seeds, the embryos and other tissues are by no means ametabolic. During maturation drying, seeds in the water content range 0.35 to 0.24g g^{-1} (between water potentials of -3 and -11MPa) have been suggested as being particularly prone to unregulated metabolism which could result in a wave of free radical generation (Vertucci and Farrant 1995). Hence the presence and optimal operation of antioxidants is seen as vital during normal physiological dehydration of orthodox seeds, and again during water uptake preparatory to germination.

In developing recalcitrant seeds metabolism is not programmed to be 'switched off': it continues, if not unabated, certainly at a relatively high level. It is thought, on the basis of some supporting evidence, that although recalcitrant seeds/embryos do possess antioxidants, these may become impaired or otherwise unable to cope with the level of free radical generation accompanying uncoordinated metabolism as a consequence of water stress (Hendry *et al.* 1992, Finch-Savage *et al.* 1993, Côme and Corbineau 1996) imposed by ongoing germinative metabolism in hydrated storage (Pammenter *et al.* 1994, Smith and Berjak 1995) or by relatively slow dehydration (Pammenter *et al.* 1998, [see later]). The evidence, although persuasive, is not consistent among recalcitrant species. This argues for differences among species in: 1. the presence of the full spectrum of antioxidants that must function as a system to be effective; 2. the qualitative and quantitative aspects of free radical production; 3. under dehydrating conditions, the rate at which water loss takes (took) place; and 4. extrinsic factors, particularly the seed-associated microflora (see later).

The presence and operation of putatively protective molecules

SUCROSE WITH CERTAIN OLIGOSACCHARIDES OR SUGAR ALCOHOLS: Maturing orthodox seeds accumulate considerable amounts of sucrose and oligosaccharides, usually raffinose and/or stachyose (Koster and Leopold 1988, Leprince *et al.* 1990, Blackman *et al.* 1992), or sucrose co-accumulates with galactosyl cyclitols (Horbowicz and Obendorf 1994, Obendorf 1997), depending on the species. As dehydration proceeds, these mixtures form a highly viscous, amorphous state, which is actually a super-saturated solution known as a glass, or described as constituting the glassy (vitrified) state (Koster and Leopold 1988, Williams and Leopold 1989, Koster 1991, Leopold *et al.* 1994, Obendorf 1997). It is not that the intracellular glassy state participates in the acquisition of desiccation-tolerance: rather, any reaction that could proceed freely in the originally fluid (hydrated) state of the cell contents, becomes increasingly curtailed as the intracellular *milieu* becomes more and more viscous (Leopold *et al.* 1994). With full development of the glassy state, there is an imposed stasis of intracellular activity, thus minimising unregulated metabolism — and its deleterious consequences. Once an orthodox seed becomes dry and ametabolic, the glassy state could play a major part in protecting membranes and curtailing events such as denaturation of macromolecules, simply by creating conditions where their attack by free radicals generated by abiotic exogenous factors (e.g. ionising radiation, including uv) is minimised because of the highly viscous intracellular environment (Pammenter and Berjak 1999). It is possible that the lifespan of mature orthodox seeds under defined storage conditions (which is species- and sometimes even variety-specific) is intimately associated with the relative stability of the glassy state, on the basis of curtailed molecular mobility (Buitink *et al.* 2000). Because they are metastable, glasses tend to break down: (crystallisation within a super-saturated sugar-based glass is a visible result of its breakdown that can be observed when a jar of honey is left to stand). This phenomenon may well underlie the inevitable, although usually slow, deterioration of orthodox seeds during storage.

On the basis that accumulation of appropriate concentrations and proportions of sucrose and oligosaccharides or galactosyl cyclitols is an essential feature of orthodox seed

survival in the desiccated state, it might be conjectured that these are lacking in recalcitrant types. This generalisation cannot, however, be made! The recalcitrant seeds of several species tested do produce sucrose and oligosaccharides (Berjak *et al.* 1989, Farrant *et al.* 1993, Finch-Savage and Blake 1994, Lin and Huang 1994, Steadman *et al.* 1996), which might accumulate in mass ratios conducive to glass formation (Horbowicz and Obendorf 1994) — but vitrification would occur only at appropriately low water contents — as is the case for maturing orthodox seeds. However, upon drying (whether 'naturally' after being shed, or experimentally after harvest) recalcitrant seeds would have lost viability at water contents considerably higher than that required for glass formation (Pammenter and Berjak 1999).

LATE EMBRYOGENIC ACCUMULATING/ABUNDANT PROTEINS (LEAs): Synthesis of the set of robust, hydrophilic proteins termed LEAs (dehydrin-like proteins), has been correlated with the peak in ABA level that occurs preceding maturation drying, during orthodox seed development (Kermode 1990). However, expression of a second set of lea genes during the drying phase when ABA levels become relatively low (Galau and Hughes 1987, Galau *et al.* 1987, reviewed by Oliver and Bewley 1997), has led to the idea that a component(s) of the signal transduction pathway other than ABA is involved in the regulation of the genes (reveiwed by Kermode 1997). Whatever the details of the control of LEA gene expression, there is convincing evidence that LEAs are somehow involved in the acquisition and maintenance of desiccation-tolerance in orthodox seeds, perhaps because their amphipathic nature facilitates interaction with a wide range of macromolecules, thus preventing denaturation of the latter under dehydrating conditions (Close *et al.* 1989, 1993, Dure *et al.* 1989, Blackman *et al.* 1995, Ingram and Bartels 1996, Close 1997). Alternative, or perhaps additional, roles suggested for LEAs is that these proteins may bind ions and water (Bray 1993, Dure 1993) or may act as chaperonins (Russouw *et al.* 1997).

The situation with respect to the occurrence and possible role of LEAs in recalcitrant seeds is equivocal. Such proteins occur in recalcitrant seeds of a variety of species, from grasses (Bradford and Chandler 1992, Gee *et al.* 1994, Still *et al.* 1994) to trees of a range of some (but not all) tropical and temperate dryland species (Finch-Savage *et al.* 1994, Gee *et al.* 1994, Farrant *et al.* 1996). On the other hand, LEAs were found to be conspicuously absent from recalcitrant seeds of ten tropical wetland species tested (Farrant *et al.* 1996). Whether or not LEAs are produced during development, the unifying feature of recalcitrant seeds is their desiccation-sensitivity. Thus the presence of these proteins alone is not sufficient to confer the ability of a seed to withstand dehydration.

The evidence of the occurrence of both appropriate sugar/oligosaccharide combinations and LEAs in recalcitrant seeds underscores the contention that no one factor can be considered to be the key factor in either the acquisition, or the maintenance, of desiccation tolerance, and the growing realisation that the phenomenon must be the result of the interplay of a variety of mechanisms and processes which will surely emerge as being under multigenic control (Pammenter and Berjak 1999).

AMPHIPATHIC SUBSTANCES: Endogenous amphipathic substances constitute a third category of putatively protective molecules: these have been suggested to partition into membrane lipid bilayers during dehydration (Hoekstra *et al.* 1997, Golovina *et al.* 1998). As a consequence, formation of the gel phase in the desiccated state is suggested to be prevented. On re-hydration, the amphipaths — e.g. flavinols (Hoekstra *et al.* 1997) — have been shown to partition back into the cytoplasm, as water becomes increasingly available. While this may well be a further mechanism involved in desiccation-tolerance in orthodox seeds, the status of amphipathic molecules in recalcitrant seeds has not yet been ascertained.

The ability for damage restitution on re-hydration

There is no doubt that although orthodox seeds are inherently protected against the exigencies of dehydration, they are damaged by a variety of commonly-used horticultural and agricultural manipulations. For example, premature harvest and forced drying at elevated temperature (as is routinely used for gymnosperm seeds) results in a syndrome of damage associated with the phenomenon of accelerated ageing (Wang *et al.* 1992). Seed storage of orthodox types under relatively high temperature and RH conditions — such as is commonly employed in warm, humid countries — has been known for many decades to affect vigour and viability adversely, which is a manifestation of damage within cells of the embryo. In fact, controlled seed treatments at elevated temperature and RH have long been used routinely to achieve accelerated ageing, which promotes such damage (e.g. Justice and Bass 1978).

The longer seeds are stored under adverse conditions (whether for agricultural or experimental purposes) and the more extreme these conditions, the greater is the accumulation of damage. However, before viability is compromised, 100% germination can still be achieved, but there is an increasing time lag between seed imbibition and radicle extension, which is the visible manifestation of germination. During this period, intracellular repair mechanisms become operational and repair must be effected before germinative metabolism can be entrained (e.g. Osborne 1983). Repair in orthodox seeds occurs at the level of protein macromolecules (Mudgett *et al.* 1997); membranes (Berjak and Villiers 1972); and nucleic acids (Elder *et al.* 1987) during the lag phase preceding germination, when it is also necessary that free-radical-scavenging systems must be restituted if damaged. In fact, the efficacy of osmopriming of low-vigour orthodox seeds resides in the facilitation of repair while the seeds are held at water potentials allowing this metabolism, but precluding the start of germination (Bray 1995).

Is the impaired ability to repair, or its lack, one of the factors contributing to seed recalcitrance? There are very few data to hand that allow assured comment to be made about this. However, following rehydration of the highly-recalcitrant seeds of *Avicennia marina*, no DNA repair is possible once 22% of the originally-present water has been lost (Boubriak *et al.* 2000). Those authors describe that, after this degree of

dehydration, the water content of *A. marina* seeds is still over 1g g^{-1} (about 53%, wmb), which argues strongly for a most inadequate DNA repair system at best, considering that this essential mechanism will operate on rehydration in orthodox seeds that have been dehydrated at least 10-fold more. In terms of free-radical scavenging processes, there is evidence that antioxidant systems fail during dehydration of desiccation-sensitive seeds and seedlings, and are assumed to remain ineffective when the water loss is made good.

Thus, although there is only a little evidence regarding the operation of repair mechanisms, including antioxidants (Côme and Corbineau 1996), in recalcitrant seeds, it is likely that these must be present in the hydrated condition, but are labile and will fail following dehydration. However, for recalcitrant seeds of individual species, the conditions under which dehydration occurs, and its extent, are likely to emerge as important determinants of the resilience of repair systems (Pammenter *et al.* 1998, Pammenter and Berjak 1999).

The mechanisms and processes outlined above, which have been implicated in acquisition and maintenance of desiccation tolerance must play an *interacting* role in the phenomenon of seed orthodoxy, but there are three important things to remember. Firstly, the list is probably by no means complete, with essential developmental phenomena remaining to be identified. Secondly, in seeds that are *not* orthodox, the features are represented to differing extents, and some may not be present at all. As a result, there are differing degrees of the extreme condition of recalcitrance, and basically of the non-orthodox behaviour of any seeds that do not complete the maturation drying phase of development, but will tolerate post-shedding dehydration to a lesser extent than will orthodox seeds. Thirdly, although seeds of individual species may survive the actual desiccation process to levels typical of orthodox seeds, if their survival is abnormally curtailed under ambient conditions, then these too must be considered as being non-orthodox.

Dehydration and effects of drying rate

Although biophysical studies have convincingly shown that intracellular water exists as at least five different states or types (Vertucci and Farrant 1995), what is necessary to understand the effects of dehydration on recalcitrant seeds, is an appreciation that in cells, water up to *c.* 0.28g g^{-1} will not freeze and is consequently different from 'bulk', freezable water. Non-freezable water is either tightly associated with (bound to) intracellular surfaces, or is glassy — and, in fact, is considered as imposing ordered structure on macromolecules, membranes and organelles. Freezable water, in contrast, may be considered as solution water (Vertucci 1990).

During maturation drying of orthodox seeds, freezable water is progressively lost, in concert with the orderly shutdown of metabolic processes and the operation of the 'checks and balances' imposed by the suite of mechanisms and processes discussed earlier (Vertucci and Farrant 1995). Below a water content of around 0.3g g^{-1}, the water removed is from the non-freezable fraction, being lost without deleterious consequences, because the embryo cells are adequately protected against the strains of extreme desiccation: this, however, is not the case for cells of recalcitrant seeds (Pammenter *et al.* 1991).

Recalcitrant seeds, which are generally relatively large structures, remain metabolic after shedding, and, because of their size and generally because they do not lose water readily, they dry slowly in nature, as well as under usual dehydrating regimes used in the laboratory. As freezable water is lost metabolism will still proceed, initially apparently normally (Berjak *et al.* 1984), but becoming unbalanced with progressively more dehydration over time (Vertucci and Farrant 1995, Pammenter *et al.* 1998, Pammenter and Berjak 1999). The more slowly water is lost, the greater is the time during which unbalanced metabolism will occur in dehydrating recalcitrant seeds — without the benefits of the suite of interacting protective mechanisms that are entrained in orthodox types. Unbalanced metabolism during prolonged periods of water stress has lethal consequences, so that the seeds generally will have lost viability by the time the water content has declined to *c.* 0.8g g^{-1} (44%, wmb) (Pammenter *et al.* 1998, Pammenter and Berjak 1999). The lethal damage that has occurred, including the consequences of uncontrolled free radical generation, is a direct consequence of unbalanced metabolism, so is termed metabolism-induced damage (Walters *et al.* 2001).

The *more rapidly* recalcitrant seeds can be dried, the relatively lower is the water content they will tolerate before viability is lost (Farrant *et al.* 1985). That is, the consequences of dehydration can be ameliorated by limiting the time for which unbalanced metabolism occurs (Pammenter *et al.* 1998, Pammenter and Berjak 1999). However, because of their inherent characteristics, even under the most severely dehydrating conditions, intact recalcitrant seeds of most species cannot be dried sufficiently rapidly to low enough water contents to exploit the limitation of metabolism-induced damage for the practical purposes of 'conventional' storage. Nevertheless, the discovery of the importance of time as a factor in the desiccation responses of recalcitrant seeds removes the obfuscation imposed by the idea of a species-specific 'critical water content' (in the range of freezable water) and has important practical consequences in terms of cryostorage (see below).

Desiccation damage of recalcitrant embryos is quite different from metabolism-induced damage. Under conditions that all, or almost all, freezable water can be removed sufficiently rapidly from recalcitrant material without compromising viability, which is possible with excised embryonic axes (Pammenter *et al.* 1991, 1993, Pritchard 1991, Finch-Savage 1992, Berjak *et al.* 1993, Pritchard and Manger 1998), the same is not true for the non-freezable water. Near, or at the stage when only non-freezable water remains, further drying rapidly induces what is referred to as desiccation-damage *sensu stricto*, which destroys cellular structures and organisation (Pammenter and Berjak 1999, Walters *et al.* 2001). The water content at which this occurs is indeed a critical water content that, within limits, may vary among recalcitrant seeds. Desiccation damage is a consequence of the absence of effective mechanisms that permit orthodox seeds to maintain viability when severely dehy-

drated. While the same may be said for metabolism-induced damage, obviously different processes and mechanisms are operative during the loss of freezable or non-freezable water.

The potential for seed storage

'Conventional' storage — i.e. use of the low temperature, low RH conditions so successful for orthodox seeds — is precluded and stored recalcitrant seeds maintain viability only when they are stored hydrated. However, this option, whether achieved in the laboratory by storage of seed monolayers in a saturated atmosphere, or use of a slightly moist packing medium (e.g. sawdust), or of a humid store, succeeds in the short-term only — which may be a few days to months, depending on the species and its provenance (Chin and Roberts 1980). Generally, viability in recalcitrant seeds of tropical species is lost extremely to relatively rapidly, while those of temperate species may last up to a year or more, particularly if they can be cold-stored. Hydrated storage therefore, is of little use for long-term conservation of the genetic resources of species producing recalcitrant seeds, and in addition to the inherently short lifespan of the seeds, the storage period is generally significantly curtailed by the activity of the associated mycoflora (Berjak 1996, Calistru *et al.* 2000). The recalcitrant-seed-associated fungi are ubiquitous, although their species composition varies seemingly from one geographic region to another (Sutherland *et al.* 2001). However, all survive on and in the seeds and the conditions of hydrated storage, especially when the seeds are chilling-sensitive, facilitate fungal proliferation which becomes rampant as the seed vigour inevitably declines. While concerted efforts are being made to overcome the major problems of hydrated storage, so as to extend post-harvest vigour and viability maximally, long-term storage must be achieved by other means.

Germplasm cryostorage

Whether they are chilling-sensitive or not, recalcitrant seeds cannot be stored at sub-zero temperatures attained by the use of conventional freezers (down to *c.* -20°C), as the slow cooling (freezing) of the necessarily-hydrated seeds results in lethal ice crystallisation. However, it is imperative that long-term conservation of the genetic resources of species producing recalcitrant (or non-orthodox) seeds be achieved. Clearly, this appears impossible (with a few exceptions — see below) using the intact seeds.

Exploitation of the finding that the more rapidly a recalcitrant seed can be dried, the relatively greater is the water loss tolerated, led to the development of and subsequent improvement in efficiency of *flash-drying* (Berjak *et al.* 1990, Wesley-Smith *et al.* 1999). This technique ensures very rapid dehydration, but not of intact seeds: instead, the embryonic axes — which generally constitute only a minute fraction of the total seed volume/mass (Berjak *et al.* 1996) — are excised and exposed to moving dry air. Flash-drying ensures that axes are rapidly dehydrated — in a matter of approximately 15min up to three hours (as opposed to days for the intact seeds), depending on the species. As the time at which uncontrolled metabolism-induced damage during drying is significantly curtailed, the axes will tolerate dehydration without loss of viability to, or close to, the level where all remaining water is non-freezable (Pammenter *et al.* 1991, 1993, Pritchard 1991, Finch-Savage 1992, Berjak *et al.* 1993, Pritchard and Manger 1998). Flash-dried axes have not become desiccation-tolerant: indeed, they will die rapidly at any ambient temperature or if refrigerated (Walters *et al.* 2001). However, they supply the material for cryostorage — i.e. storage at temperatures below -80°C, but conventionally in/over liquid nitrogen (-196°C or *c.* -150°C, respectively).

Successful cryostorage — which will result in the production of normal seedlings — is, however, not straightforward, and indeed, the literature records far more failure than success (Berjak *et al.* 1996). The parameters, especially of cooling (freezing) rate and thawing conditions presently have to be ascertained on a species basis. It is also imperative that the preliminary flash-drying be optimised to ensure that only as *little* water as is necessary, is removed, as the closer the final hydration level is to non-freezable water only, the more the axes become stressed (Wesley-Smith *et al.* 1992, 1999).

Cooling parameters must ensure, that at whatever the water content of the axes, the formation of large, potentially lethal intracellular ice crystals is curtailed. Generally, the higher the final water content after flash-drying, the more rapid must be the passage of the axes through the temperature range permitting ice crystallisation, which demands very rapid cooling (Wesley-Smith *et al.* 1992). Additionally, the choice of cryogen (coolant) is also important, as it must cause no biological injury but must also be a good conductor of heat away from the axis during its plunge (Wesley-Smith *et al.* 1999). Cooling rates can be slower only in line with low axis water contents, which themselves, may be injurious. Whatever the cryogen used for the cooling process, liquid nitrogen is generally used for cryostorage. Once axes are retrieved from cryostorage, thawing conditions are also critical, as ice crystallisation readily occurs during warming through the critical temperature range. An additional complication is the choice of the medium in which thawing is carried out: the axes are dehydrated, so thawing is not merely a matter of their reaching ambient temperature, but also of their rehydration (Berjak *et al.* 1999b).

An over-arching complication of axis cryostorage is that germination must be achieved under *in vitro* conditions, for which the medium that replaces the normal nutrient and water supply of the seed must be optimised *before* anything else can be attempted. Additionally, all inoculum of the ubiquitous seed-associated micro-organisms (generally fungi) must have been totally eliminated, as these not only will over-run axes in culture, but survive flash-drying and cryostorage remarkably well (Berjak *et al.* 1999a).

Despite all these complications, it seems that there is no alternative to cryostorage for long-term conservation of the genetic resources of species producing recalcitrant seeds and notable success has been achieved for several temperate and tropical species. Nevertheless, at the time of writing, there are species which appear to be intractable to the necessary manipulations (Kioko *et al.* 1998, Berjak *et al.*

1999c). However, because of the inherent variability of recalcitrant seeds (Berjak *et al.* 1989, 1996), in these cases additional parameters, such as seed developmental status in the newly-harvested condition, need to be related to the response of the axes. Then, for those species where the embryonic axis is too large to be manipulated at all, alternative explants, e.g. somatic embryos or apical meristems, need to be considered.

Ideally, intact seeds should be cryostored, which would obviate *in vitro* complications: however, size precludes this in the great majority of cases, although there are a few species that produce seeds which are sufficiently small. For success, such seeds must be capable of rapid dehydration. Notably, successful cryostorage has been achieved for the apparently non-orthodox seeds of *Warburgia salutaris* and *W. ugandensis* (Kioko *et al.* 1999, 2000). The former species, which is arguably the most sought-after (for traditional medicinal uses) and consequently the most threatened in South Africa, produces fruits that are highly insect-predated, so effectively, mature seeds are seldom obtained. When precautions against insect attack are taken, the seeds — and those of *W. ugandensis* — lose viability if subjected to slow air-drying. Their successful cryostorage is a major step in conserving the biodiversity of the genus — and, in fact, ensuring its survival for *W. salutaris*.

References

Bain J, Mercer FV (1968) Subcellular organisation of the developing cotyledons of *Pisum sativum* L. Australian Journal of Biological Sciences **19**: 49–67

Berjak P (1996) The role of micro-organisms in deterioration during storage of recalcitrant and intermediate seeds. In: Ouédraogo A-S, Poulsen K, Stubsgaard I (eds) Intermediate/Recalcitrant Tropical Forest Seeds, IPGRI, Rome, pp 121–126

Berjak P, Pammenter NW (1994) Recalcitrance is not an all-or-nothing situation. Seed Science Research **4**: 263–264

Berjak P, Villiers TA (1972) Ageing in plant embryos II. Age-induced damage and its repair during early germination. New Phytologist **71**: 135–144

Berjak P, Dini M, Pammenter NW (1984) Possible mechanisms underlying the differing dehydration responses in recalcitrant and orthodox seeds: desiccation-associated subcellular changes in propagules of *Avicennia marina*. Seed Science and Technology **12**: 365–384

Berjak P, Farrant JM, Pammenter NW (1989) The basis of recalcitrant seed behaviour. In: Taylorson RB (ed.) Recent Advances in the Development and Germination of Seeds. Plenum Press, New York, pp 89–108

Berjak P, Vertucci CW, Pammenter NW (1993) Effects of developmental status and dehydration rate on characteristics of water and desiccation-sensitivity in recalcitrant seeds of *Camellia sinensis*. Seed Science Research **3**: 155–166

Berjak P, Farrant JM, Mycock DJ, Pammenter NW (1990) Recalcitrant (homoiohydrous) seeds: the enigma of their desiccation-sensitivity. Seed Science and Technology **18**: 297–310

Berjak P, Mycock DJ, Wesley-Smith J, Dumet D, Watt MP (1996) Strategies for *in vitro* conservation of hydrated germplasm. In: Normah MN, Narimah MK, Clyde MM (eds) *In vitro* Conservation of Plant Genetic Resources. Percetakan Watan Sdn. Bhd, Kuala Lumpur, Malaysia, pp 19–52

Berjak P, Walker M, Watt MP, Mycock DJ (1999a) Experimental parameters underlying failure or success in plant germplasm cryopreservation. A case study on zygotic axes of *Quercus robur* L. CryoLetters **20**: 251–262

Berjak P, Mycock DJ, Walker M, Kioko JI, Pammenter NW, Wesley-Smith J (1999b) Conservation of genetic resources naturally occurring as recalcitrant seeds. In: Black M, Bradford KJ, Vázqez-Ramos J (eds) Seed Biology — Advances and Applications. CABI Publishing, Wallingford, UK, pp 223–228

Berjak P, Kioko JI, Walker M, Mycock DJ, Wesley-Smith J, Watt P, Pammenter NW (1999c) Cryopreservation — an elusive goal? In: Marzalina M, Khoo KC, Jayanthi N, Tsan FY, Krishnapillay, B (eds): Recalcitrant Seeds, FRIM, Malaysia, pp 96–109

Blackman SA, Obendorf RL, Leopold AC (1992) Maturation proteins and sugars in desiccation tolerance of developing soybean seeds. Plant Physiology **100**: 225–230

Blackman SA, Obendorf RL, Leopold AC (1995) Desiccation tolerance in developing soybean seeds: the role of stress proteins. Physiologia Plantarum **93**: 630–638

Boubriak I, Dini M, Berjak P, Osborne DJ (2000) Desiccation and survival in the recalcitrant seeds of *Avicennia marina*: DNA replication, DNA repair and protein synthesis. Seed Science Research **10**: 307–315

Bradford KJ, Chandler PM (1992) Expression of dehydrin-like proteins in embryos and seedlings of *Zizania palustris* and *Oryza sativa* during dehydration. Plant Physiology **99**: 488–494

Bray CM (1995) Biochemical processes during the osmopriming of seeds. In: Kigel J, Galili G (eds) Seed Development and Germination. Marcel Dekker Inc., New York, pp 767–789

Bray E (1993) Molecular responses to water deficit. Plant Physiology **103**: 1035–1040

Brunori A (1967) A relationship between DNA synthesis and water content during ripening of *Vicia faba* seeds. Caryologia **20**: 333–338

Buitink J, Hoekstra FA, Hemminga MA (2000) Molecular mobility in the cytoplasm of lettuce radicles correlates with longevity. Seed Science Research **10**: 285–292

Calistru C, McLean M, Pammenter NW, Berjak P (2000) The effects of mycofloral infection on the viability and ultrastructure of wet-stored seeds of *Avicennia marina* (Forssk.) Vierh. Seed Science Research **10**: 341–353

Chin HF, Roberts EH (1980) Recalcitrant Crop Seeds. Tropical Press SDN.BDH, Kuala Lumpur, Malaysia

Chien C-T, Lin T-P (1997) Effect of harvest date on the storability of desiccation-sensitive seeds of *Machilus kusanoi* Hay. Seed Science and Technology **25**: 361–371

Close TJ (1997) Dehydrins: a commonality in the response of plants to dehydration and low temperature. Physiologia Plantarum **100**: 291–296

Close TJ, Kortt AA, Chandler PM (1989) A cDNA-based comparison of dehydration-induced proteins (dehydrins) in barley and corn. Plant Molecular Biology **13**: 95–108

Close TJ, Fenton RD, Yang A, Asghar R, DeMason DA, Crone DE, Meyer NC, Moonan F (1993) Dehydrin: the protein. In: Close TJ, Bray EA (eds) Current topics in plant physiology, Vol. 10. Plant responses to cellular dehydration during environmental stress. American Society of Plant Physiologists, Rockville, pp 104–119

Côme D, Corbineau F (1996) Metabolic damage related to desiccation sensitivity. In: Ouédraogo A-S, Poulsen K, Stubsgaard F (eds) Intermediate/Recalcitrant Tropical Forest Tree Seeds. IPGRI, Rome, pp 83–97

Drew PJ, Pammenter NW, Berjak P (2000) 'Sub-imbibed' storage is not an option for extending longevity of recalcitrant seeds of the tropical species, *Trichilia dregeana* Sond. Seed Science Research **10**: 355–363

Dure L III (1993) A repeating 11-mer amino acid motif and plant desiccation. Plant Journal **3**: 363–369

Dure L III, Crouch M, Harada J, Ho THD, Mundy J, Quatrano R, Thomas T, Sung ZR (1989) Common amino acid sequence

Molecular Biology **12**: 475–486
Elder RH, Dell'Aquila A, Mezzina M, Sarasin A, Osborne DJ (1987) DNA ligase in repair and replication in the embryos of rye, *Secale cereale*. Mutation Research **181**: 61–71
Ellis RH, Roberts EH (1980) Improved equations for the prediction of seed longevity. Annals of Botany **45**: 13–30
Ellis RM, Hong TD, Roberts EH (1990) An intermediate category of seed storage behaviour? I. Coffee. Journal of Experimental Botany **41**: 1167–1174
Farrant JM, Berjak P, Pammenter NW (1985) The effect of drying rate on viability retention of recalcitrant propagules of *Avicennia marina*. South African Journal of Botany **51**: 432–438
Farrant JM, Pammenter NW, Berjak P (1986) The increasing desiccation sensitivity of recalcitrant *Avicennia marina* seeds with storage time. Physiologia Plantarum **67**: 291–298
Farrant JM, Pammenter NW, Berjak P (1989) Germination-associated events and the desiccation sensitivity of recalcitrant seeds — a study on three unrelated species. Planta **178**: 189–198.
Farrant JM, Pammenter NW, Berjak P (1992) Development of the recalcitrant (homoiohydrous) seeds of *Avicennia marina*: Anatomical, ultrastructural and biochemical events associated with development from histodifferentiation to maturation. Annals of Botany **70**: 75–86
Farrant JM, Pammenter NW, Berjak P (1993) Seed development in relation to desiccation tolerance: A comparison between desiccation-sensitive (recalcitrant) seeds of *Avicennia marina* and desiccation-tolerant types. Seed Science Research **3**: 1–13
Farrant JM, Pammenter NW, Berjak P, Walters C (1997) Subcellular organization and metabolic activity during the development of seeds that attain different levels of desiccation tolerance. Seed Science Research **7**: 135–144
Farrant JM, Pammenter NW, Berjak P, Farnsworth EJ, Vertucci CW (1996) Presence of dehydrin-like proteins and levels of abscisic acid in recalcitrant (desiccation sensitive) seeds may be related to habitat. Seed Science Research **6**: 175–182
Finch-Savage WE (1992) Seed water status and survival in the recalcitrant species *Quercus robur* L.: Evidence for a critical moisture content. Journal of Experimental Botany **43**: 671–679
Finch-Savage, WE (1996) The role of developmental studies in research on recalcitrant and intermediate seeds In: Ouédraogo A-S, Poulsen K, Stubsgaard F (eds) Intermediate/Recalcitrant Tropical Forest Tree Seeds. IPGRI, Rome, pp 83–97
Finch-Savage WE, Blake PS (1994) Indeterminate development in desiccation-sensitive seeds of *Quercus robur* L. Seed Science Research **4**: 127–133
Finch-Savage WE, Pramanik SK, Bewley JD (1994) The expression of dehydrin proteins in desiccation-sensitive (recalcitrant) seeds of temperate trees. Planta **193**: 478–485
Finch-Savage WE, Grange RI, Hendry GAF, Atherton NM (1993) Embryo water status and loss of viability during desiccation in the recalcitrant seed species *Quercus robur* L. In: Côme D, Corbineau F (eds) Fourth International Workshop on Seeds: Basic and Applied Aspects of Seed Biology. AFSIS, Paris, pp 723–730
Finch-Savage WE, Bergervoet JHW, Bino RJ, Clay HA, Groot SPC (1998) Nuclear replication activity during seed development, dormancy breakage and germination in three tree species: Norway maple (*Acer platanoides* L.), sycamore (*Acer pseudoplatanus* L.) and cherry (*Prunus avium* L.). Annals of Botany **81**: 519–526
Fu J-R, Jin JP, Peng YF, Xia QH (1994) Desiccation tolerance in two species with recalcitrant seeds: *Clausena lansium* (Lour.) and *Litchi chinensis* (Sonn.). Seed Science Research **4**: 257–261
Galau GA, Hughes DW (1987) Coordinate accumulation of homologous transcripts of seven *lea* gene families during embryogenesis and germination. Developmental Biology **123**: 213–221
Galau GA, Bijaisoradat N, Hughes DW (1987) Accumulation kinetics of cotton late embryogenesis-abundant mRNAs: coordinate regulation during embryogenesis and the role of abscisic acid. Developmental Biology **123**: 198–212
Gee OH, Probert RJ, Coomber SA (1994) 'Dehydrin-like' proteins and desiccation tolerance in seeds. Seed Science Research **4**: 135–141
Golovina EA, Hoekstra FA, Hemminga MA (1998) Drying increases intracellular partitioning of amphiphilic substances into the lipid phase: impact on membrane permeability and significance for desiccation tolerance. Plant Physiology **118**: 975–986
Hallam ND (1972) Embryogenesis and germination in rye (*Secale cereale*). I. Fine structure of the developing embryo. Planta **104**: 157–166
Hendry GAF (1993) Oxygen, free radical processes and seed longevity. Seed Science Research **3**: 141–153
Hendry GAF, Finch-Savage WE, Thorpe PC, Atherton NM, Buckland SM, Nilsson KA, Seel WA (1992) Free radical processes and loss of viability during desiccation in the recalcitrant species *Quercus robur* L. New Phytologist **122**: 273–279
Hoekstra FA, Wolkers WF, Buitink J, Golovina EA, Crowe JH, Crowe LM (1997) Membrane stabilization in the dry state. Comparative Biochemistry and Physiology **117A**: 335–341
Hong TD, Ellis RH (1990) A comparison of maturation drying, germination, and desiccation tolerance between developing seeds of *Acer pseudoplatanus* L. and *Acer platenoides* L. New Phytologist **116**: 589–596
Hong TD, Ellis RM (1996) A Protocol to Determine Seed Storage Behaviour. Rome, IPGRI
Horbowicz M, Obendorf RL (1994) Seed desiccation tolerance and storability: Dependence on flatulence-producing oligosaccharides and cyclitols — review and survey. Seed Science Research **4**: 385–405
Ingram I, Bartels D (1996) The molecular basis of dehydration tolerance in plants. Annual Review of Plant Physiology and Plant Molecular Biology **47**: 377–403
Justice OL, Bass LN (1978) Principles and Practices of Seed Storage. Agricultural Handbook number 506, United States Department of Agriculture
Kermode AR (1990) Regulatory mechanisms involved in the transition from seed development to germination. Critical Reviews in Plant Science **9**: 155–195
Kermode AR (1997) Approaches to elucidate the basis of desiccation-tolerance in seeds. Seed Science Research **7**: 75–95
Kioko J, Berjak P, Pritchard H, Daws M (1999) Studies of post-shedding behaviour and cryopreservation of seeds of *Warburgia salutaris*, a highly-endangered medicinal plant indigenous to tropical Africa. In: Marzalina M, Khoo KC, Jayanthi N, Tsan FY, Krishnapillay B (eds) Recalcitrant Seeds. FRIM, Kuala Lumpur, Malaysia, pp 365–371
Kioko JI, Berjak P, Pritchard HW, Daws M (2000) Seeds of the African pepperbark (*Warburgia salutaris*) can be cryopreserved after rapid dehydration in silica gel. In: Engelmann F, Takagi H (eds) Cryopreservation of Tropical Plant Germplasm — Current Research Progress and Applications. IPGRI, Rome, pp 371–377
Kioko JI, Berjak P, Pammenter NW, Watt P, Wesley-Smith J (1998) Desiccation and cryopreservation of embryonic axes of *Trichilia dregeana* Sond. *CryoLetters* **19**: 5–11
Klein S, Pollock BM (1968) Cell fine structure of developing lima bean seeds related to seed desiccation. American Journal of Botany **55**: 658–672
Koster KL (1991) Glass formation and desiccation tolerance in seeds. Plant Physiology **96**: 302–304
Koster KL, Leopold AC (1988) Sugars and desiccation tolerance in seeds. Plant Physiology **88**: 829–832
Leopold AC, Sun WQ, Bernal-Lugo I (1994) The glassy state in seeds: analysis and function. Seed Science Research **4**:

267–274
Leprince O, Bronchart R, Deltour R (1990) Changes in starch and soluble sugars in relation to the acquisition of desiccation tolerance during maturation of *Brassica campestris* seeds. Plant Cell and Environment **13**: 539–546
Lin T-P, Chen M-H (1995) Biochemical characteristics associated with the development of the desiccation-sensitive seeds of *Machilus thunbergii* Sieb. & Zucc. Annals of Botany **76**: 381–387
Lin T-P, Huang N-H (1994) The relationship between carbohydrate composition of some tree seeds and their longevity. Journal of Experimental Botany **45**: 1289–1294
Obendorf RL (1997) Oligosaccharides and galactosyl cyclitols in seed desiccation tolerance. Seed Science Research **7**: 63-74
Osborne DJ, Boubriak II (1994) DNA and desiccation tolerance. Seed Science Research **4**: 175–185
Moreno Díaz de la Espina S (1995) Nuclear matrix isolated from plant cells. International Review of Cytology **162B**: 75–139
Mudgett MB, Lowensen JD, Clarke S (1997) Protein repair L-isoaspartyl methyltransferase in plants. Phylogenetic distribution and the accumulation of substrate proteins in aged barley seeds. Plant Physiology **115**: 1481–1489
Mumford PM, Brett AC (1982) Conservation of cacao seeds. Tropical Agriculture **59**: 306–310
Oliver MJ, Bewley JD (1997) Desiccation-tolerance of plant tissues: A mechanistic overview. Horticultural Reviews **18**: 171–213
Osborne DJ (1983) Biochemical control of systems operating in the early hours of germination. Canadian Journal of Botany **61**: 3568–3577
Poulsen KN, Eriksen EN (1992) Physiological aspects of recalcitrance in embryonic axes of *Quercus robur* L. Seed Science Research **2**: 215–221
Pammenter NW, Berjak P (1999) A review of recalcitrant seed physiology in relation to desiccation-tolerance mechanisms. Seed Science Research **9**: 13–37
Pammenter NW, Berjak P (2000) Evolutionary and ecological aspects of recalcitrant seed biology. Seed Science Research **10**: 301–306
Pammenter NW, Vertucci CW, Berjak P (1991) Homeohydrous (recalcitrant) seeds: dehydration, the state of water and viability characteristics in *Landolphia kirkii*. Plant Physiology **96**: 1093–1098
Pammenter NW, Vertucci CW, Berjak P (1993) Responses of dehydration in relation to non-freezable water in desiccation-sensitive and -tolerant seeds. In: Côme D, Corbineau F (eds) Fourth International Workshop on Seeds: Basic and Applied Aspects of Seed Biology. ASFIS, Paris, pp 867–872
Pammenter NW, Berjak P, Farrant JM, Smith MT, Ross G (1994) Why do stored, hydrated recalcitrant seeds die? Seed Science Research **4**: 187–191
Pammenter NW, Greggains V, Kioko JI, Wesley-Smith J, Berjak P, Finch-Savage WE (1998) Effects of differential drying rates on viability retention of recalcitrant seeds of *Ekebergia capensis*. Seed Science Research **8**: 463–471
Pritchard HW (1991) Water potential and embryonic axis viability in recalcitrant seeds of *Quercus rubra*. Annals of Botany **67**: 43–49
Pritchard HW, Manger KR (1998) A calorimetric perspective on desiccation stress during preservation procedures with recalcitrant seeds of *Quercus robur* L. CryoLetters **19 (Supplement 1)**: 23–30
Pritchard HW, Tompsett PB, Manger K, Smidt WJ (1995) The effect of moisture content on the low temperature response of *Araucaria huntsteinii* seed and embryos. Annals of Botany **76**: 79–88
Roberts EH (1973) Predicting the storage life of seeds. Seed Science and Technology **1**: 499–514
Rogerson NE, Matthews S (1977) Respiratory and carbohydrate changes in developing pea (*Pisum sativum*) seeds in relation to their ability to withstand desiccation. Journal of Experimental Botany **28**: 304–313
Russouw PS, Farrant JM, Brandt W, Lindsey GG (1997) The most prevalent protein in a heat-treated extract of pea (*Pisum sativum*) is a LEA group I protein; its conformation is not affected by exposure to high temperature. Seed Science Research **7**: 117–123
Sacandé M, Groot SPC, Hoekstra FA, De Castro RD, Bino RJ (1997) Cell cycle events in developing neem (*Azadirachta indica*) seeds: are they related to intermediate storage behaviour? Seed Science Research **7**: 161–168
Smith MT, Berjak P (1995) Deteriorative changes associated with the loss of viability of stored desiccation-tolerant and -sensitive seeds. In: Kigel J, Galili G (eds) Seed Development and Germination. Marcel Dekker Inc, New York, pp 701–746
Steadman KJ, Pritchard HW, Dey PM (1996) Tissue-specific soluble sugars in seeds as indicators of storage category. Annals of Botany **77**: 667–674
Still DW, Kovach DA, Bradford KJ (1994) Development of desiccation tolerance in rice (*Oryza sativa*) and wild rice (*Zizania palustris*). Dehydrin expression, abscisic acid content, and sucrose accumulation. Plant Physiology **104**: 431–438
Sutherland JR, Diekmann M, Berjak P (2001) Forest Tree Seed Health Testing. IPGRI, Rome (in press)
Suszka B, Tylkowski T (1980) Storage of acorns of the English oak (*Quercus robur* L.) over 1–5 winters. Arboretum Kórnickie **25**: 199–229
Tompsett PB, Pritchard HW (1993) Water status changes during development in relation to the germination and desiccation tolerance of *Aesculus hippocastanum* L. seeds. Annals of Botany **71**: 107–116
Vertucci CW (1990) Calorimetric studies on the state of water in seed tissues. Biophysical Journal **58**: 1463–1471
Vertucci CW, Farrant JM (1995) Acquisition and loss of desiccation tolerance. In: Kigel J, Galili G (eds) Seed Development and Germination. New York, Marcel Dekker Inc., New York, pp 237–271
Walters C (ed.) (1998) Ultra-dry seed storage. Seed Science Research 8 (Supplement No. 1)
Walters C, Pammenter NW, Berjak P, Crane J (2001) Desiccation damage, accelerated ageing and respiration in desiccation tolerant and sensitive seeds. Seed Science Research **11**: 135–148
Wang BSP, Downie B, Wetzel S, Palamarek D, Hamilton R (1992) Effects of cone scorching on germinability and vigour of lodgepole pine (*Pinus contorta* var. *latifolia*) seeds in Alberta. Seed Science and Technology **20**: 409–419
Webb MA, Arnott HJ (1982) Cell wall conformation in dry seeds in relation to the preservation of structural integrity during desiccation. American Journal of Botany **69**: 1657–1668
Wesley-Smith J, Vertucci C, Berjak P, Pammenter NW, Crane J (1992) Cryopreservation of desiccation-sensitive axes of *Camellia sinensis* in relation to dehydration, freezing rate and the thermal properties of tissue water. Journal of Plant Physiology **140**: 596–604
Wesley-Smith J, Walters C, Berjak P, Pammenter NW (1999). A method for the cryopreservation of embryonic axes at ultra-rapid cooling rates. In Marzalina M, Khoo KC, Jayanthi N, Tsan FY, Krishnapillay, B (eds) Recalcitrant Seeds, FRIM, Malaysia, pp 132–139
Williams RJ, Leopold AC (1989) The glassy state in corn embryos. Plant Physiology **89**: 977–981
Wolfe SL (1993) Molecular and Cellular Biology. Wadsworth, Belmont, California

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